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Mechanisms regulating angiogenesis underlie seasonal control of pituitary function

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Abstract

Seasonal changes in mammalian physiology, such as those affecting reproduction, hibernation and metabolism, are controlled by pituitary hormones released in response to annual environmental changes. In temperate zones, the primary environmental cue driving seasonal reproductive cycles is the change in daylength (photoperiod), encoded by the pattern of melatonin secretion from the pineal gland. However, although reproduction relies on hypothalamic gonadotrophin-releasing hormone (GnRH) output, and most cells producing reproductive hormones are in the pars distalis (PD) of the pituitary, melatonin receptors are localized in the pars tuberalis (PT), a physically and functionally separate part of the gland. How melatonin in the PT controls the PD is not understood. Here we show that melatonin time-dependently acts on its receptors in the PT to alter splicing of vascular endothelial growth factor (VEGF). Outside the breeding season, angiogenic VEGF-A stimulates vessel growth in the infundibulum, aiding vascular communication between the PT, PD and brain. This also acts on VEGFR2 expressed in PD prolactin-producing cells known to impair gonadotrophin secretion. In contrast, in the breeding season, melatonin releases anti-angiogenic VEGF-A_{xxx}b from the PT, inhibiting infundibular angiogenesis and diminishing lactotroph VEGFR2 expression, lifting reproductive axis repression in response to shorter day lengths. The time-dependent, melatonin-induced differential expression of VEGF-A isoforms culminates in alterations in gonadotroph function opposite to those of lactotrophs, with up-regulation and

down-regulation of gonadotropin gene expression during the breeding and non-breeding season, respectively. These results provide a novel mechanism by which melatonin can control pituitary function in a seasonal manner.

Introduction

Pituitary hormone secretion regulates multiple functions in the body, including fertility, growth, fluid balance and the response to stress. This regulation displays annual oscillations in most mammalian species, and is overtly seasonal in animals that have a tightly controlled reproductive window. It is thought that both endogenous (circadian and circannual rhythm generators) and exogenous (photoperiod) cues can contribute to drive seasonal physiology [1]. The duration of nocturnal release of the pineal hormone melatonin underlies the photoperiodic control of seasonality in sheep [2]. As the synthesis and release of melatonin is inhibited by light, the longer nights of winter are associated with longer duration of melatonin production, whereas the opposite is true in the summer. Even though the reproductive cycle relies primarily on the secretion of gonadotropin-releasing hormone (GnRH) from the hypothalamus [3], one of the major sites of melatonin action is the pars tuberalis (PT) of the pituitary gland [4, 5]. Indeed, melatonin is a regulator of endocrine function in the pituitary, and specifically inhibits prolactin-producing lactotrophs, known to be associated with repression of the reproductive cycle, directly within this tissue [6]. However, the mechanism through which melatonin exerts this influence remains unclear.

Three functionally distinct regions of the pituitary gland, the PT, pars distalis (PD) and infundibulum intercommunicate with one another via an elegant portal vascular arrangement. Although melatonin is known to act on its receptors in the PT, lactotrophs – the cells inhibited by melatonin, are exclusively found in the PD

[7, 8]. The mechanisms through which melatonin, acting in the PT, can control pituitary function in the PD are unresolved. Here we describe how the vascular arrangement in the infundibulum of the pituitary was drastically altered between the breeding and non-breeding seasons (BS and NBS, respectively) in sheep. We therefore tested the hypothesis that melatonin could act to regulate pituitary function through the control of blood vessel growth and communication between the PT and PD.

Blood vessel growth (angiogenesis) is regulated by vascular endothelial growth factors (VEGF), a family of peptides of which the most potently angiogenic and widely expressed is VEGF-A. Multiple VEGF-A products can be generated by alternative splicing of a single gene [9, 10]. Alternative use of exons 6 and 7 will result in proteins of different length (e.g. 120, 164 or 188 amino acids in sheep, and 121, 165 or 189 in human). Use of the proximal splice site in exon 8 generates the pro-angiogenic VEGF-A_{xxx}a isoforms, and use of the distal splice site in exon 8 generates the VEGF-A_{xxx}b isoforms, where xxx denotes the number of amino acids, and a or b denotes the carboxy-terminal amino acid sequence. The most extensively studied and highly expressed isoforms of each family (VEGF-A_{165}b and VEGF-A_{165}a, respectively) are able to counteract the effects of each other on blood vessel growth [11]. When VEGF-A_{165}a binds VEGFR2 on endothelial cells it causes robust autophosphorylation and downstream signalling through phospholipase C, src, ras and other pathways to induce a raft of responses including angiogenesis, vasodilatation, increased vascular permeability and cytoprotection [12].

Significance Statement

Adaptation to seasonal changes in the environment is critical for survival in all species. In vertebrates, annual oscillations in pituitary hormones underlie the regulation of seasonal physiology. We found that, in sheep, the duration of pineal melatonin output at night controls the production of different forms of the protein, vascular endothelial growth factor (VEGF) within a specific pituitary region, the pars tuberalis. Forms that block blood-vessel growth are made in winter, but those that stimulate it are made in summer. Further to the resulting remodelling of the vascular connection between the brain and pituitary, the temporally divergent VEGF-A variants operate as messenger signals on endocrine cells of a different part of the gland, the pars distalis, to regulate seasonal fertility.

In contrast, although the binding affinity for VEGFR2 is the same as that of VEGF-A_{165}a, VEGF-A_{165}b induces weak phosphorylation [13], does not bind the co-receptor Neuropilin-1 [14], which is responsible for intracellular trafficking and recycling to the membrane [15], and does not activate the full signalling pathway. This means it does not induce angiogenesis or vasodilatation [11], or a sustained increase in vascular permeability [16], and results in VEGFR2 degradation, not recycling [15]. It does, however, stimulate cytoprotection of endothelial and epithelial cells [17] and neurons [18]. These two isoform families, therefore, have very different physiological consequences [19], but any differential role in seasonal pituitary angiogenesis is unknown. We found that while total VEGF-A was not altered between the BS and NBS, there was a dramatic switch in splicing

in the BS from angiogenic VEGF-A_{xxx}a isoforms to anti-angiogenic VEGF-A_{xxx}b isoforms in both the PT and PD. This was mirrored by a reduction in the number of blood vessels in the infundibulum. Melatonin receptor expression in the PT and the infundibulum co-localized with cells expressing VEGF-A. Two potential mechanisms for VEGF-A-mediated regulation of pituitary seasonality are proposed here – a) VEGF-A acting to regulate blood vessel function, which subsequently controls delivery of other hormones to the PD, and b) VEGF-A acting directly on lactotrophs to control prolactin–associated down-regulation of the reproductive axis. We found that VEGFR2 co-localization with prolactin in the PD was increased outside the BS, consistent with a VEGF-A_{xxx}a mediated repression of fertility. In vitro culture of PT cells from BS sheep showed that duration of melatonin exposure controlled VEGF-A isoform secretion – long exposure induced VEGF-A_{xxx}b production, whereas short exposure induced VEGF-A_{xxx}a production. Culture of PT cells from the NBS revealed that melatonin given at frequencies seen in the winter could switch the expression of VEGF-A isoforms to BS levels. We then showed that PT cells treated with NBS melatonin regimens release VEGF-A_{xxx}a, which directly induced prolactin secretion from PD cells. Finally, the time-dependent, melatonin-induced differential expression of VEGF-A isoforms resulted in alterations of gonadotroph function opposite to those of lactotrophs in each season. Together, these results demonstrate that melatonin-mediated control of VEGF splicing could underlie intra-pituitary regulation of seasonal fertility.

Figure 1. Angiogenesis in the pituitary is seasonally dependent. A. Endothelial staining in the pars tuberalis (PT)/infundibulum and quantification of vessel loops in the summer (non-breeding season, NBS) and winter (breeding season, BS). B. Endothelial proliferation (PCNA/CD31 double positive cells) in the NBS and BS. C. Total VEGF-A levels in the PT/stalk in the BS and NBS (not significantly different; $p>0.05$). D. VEGF-Axxxb specific ELISA on protein extracted from pituitaries of animals killed in the BS or NBS. E. Proportion of VEGF-A that was VEGF-Axxxb in the BS and NBS. F. Staining of the melatonin receptor (green) and VEGF-A (red) in different regions of the pituitary; co-staining (found in PT and vascular loops) is shown as yellow. ***= $p<0.01$, ***=$p<0.001$ compared with BS. Scale bar = 50μm

Results

Vascular growth in the pituitary gland is seasonally controlled

To investigate the vascular architecture of the pituitary in a seasonally breeding mammal, we screened pituitary glands of sheep with the endothelial marker CD31. Staining showed a significant ($p<0.001$) increase in the number of vascular loops extending from the PT into the infundibulum in the summer (non-breeding season, NBS) compared with animals culled in the winter (breeding season, BS; Figure 1A). To determine whether this was due to endothelial proliferation in the NBS, we co-stained for CD31 and proliferating cell nuclear antigen, PCNA. We detected proliferating endothelial cells in both seasons, but a two-fold increase in proliferating endothelial cells in the NBS (Figure 1B). As angiogenesis is driven by VEGF, we measured VEGF-A in the pars-tuberalis/stalk region of the pituitary. There was no difference in VEGF-A as measured by antibodies that detect all isoforms of VEGF-A (panVEGF; Figure

1C). However, using antibodies that specifically detect the exon 8b splice variants (VEGF-A_{xxx}b), the expression of which has been shown to be anti-angiogenic *in vivo*, a reduction in VEGF-A_{xxx}b was measured in the NBS (Figure 1D). This resulted in a change in the ratio of VEGF-A from 33% excess anti-angiogenic isoforms in the BS, to 60% excess angiogenic isoforms in the NBS (Figure 1E). This indicates that the pituitary is in an anti-angiogenic state in the BS, and suggests a link between day length and VEGF-A splicing. To determine whether VEGF-A was expressed in the pituitary in cells that can respond to day length, we co-stained for melatonin receptor and VEGF. Figure 1F shows that MT1 and VEGF-A were co-localized in the PT and, interestingly, also in the vascular loops (arrows) that connect the PT with the infundibulum. In contrast, while VEGF-A was expressed in the PD, MT1 receptors were not. The anti-angiogenic isoforms have not previously been cloned from sheep, so we examined RNA expression by RT-PCR. Both isoforms were detected in pituitaries from sheep in both seasons (PD and PT, supplementary Figure 1A). Cloning and sequencing of the PCR product confirmed that this was sheep VEGF-A_{xxx}b (supplementary Figure 1B). The sheep sequence has a single nucleotide substitution (a G in sheep, C in human) compared with human DNA. This results in a single amino acid difference, with a sequence of SRTRKD instead of SLTRKD in human. Thus, the sheep VEGF-A_{xxx}b isoforms are one amino acid shorter than the human ones. The cell type in which VEGF-A_{xxx}b was expressed in the PT was identified by immunolocalization. Supplementary Figure 1C confirms that VEGF-A_{xxx}b is expressed in the MT1 positive cells, which, in the

PT, are not endothelial or glial-type folliculostellate (S100+) cells. These results suggested that melatonin could regulate expression of different VEGF-A isoforms in the PT, so regulating angiogenesis in the pituitary in a seasonally dependent manner.

VEGF-A splicing is regulated by duration of melatonin exposure in PT cells

We investigated VEGF-A isoform expression in cells isolated from the PT, which express both the melatonin receptor and VEGF-A (supplementary Figure 2A) by isoform family specific ELISA and real time PCR. Cells were cultured under conditions without melatonin (control, supplementary Figure 2B) or with melatonin given for 16hrs (mimicking winter –BS regimen), or 8 hours (mimicking summer –NBS regimen). VEGF-A_{xxx}b protein (Figure 2A) was increased 7 fold by a BS melatonin regimen (Figure 2A) in cells from BS animals (i.e. given the matching melatonin regimen), whereas panVEGF-A increased only 4 fold (Figure 2B). In contrast, when cells from pituitaries of NBS sheep were given the NBS melatonin regimen, VEGF-A_{xxx}b was only increased 2-fold (significantly lower than BS, Figure 2A), but panVEGF-A was increased 5-fold (Figure 2B), although this response took longer than in the BS cells. This suggests that the length of time that the cells are daily exposed to melatonin controls the expression of the different splice variants of VEGF-A. It also suggests that NBS cells are less prepared to respond to melatonin exposure, as they take longer to increase their VEGF-A_{xxx}a output. To determine whether this was dependent on the stage of the annual reproductive cycle, we treated cells with a melatonin regimen that was

reversed (i.e. opposite to that of the prevailing photoperiod) and found the same response for VEGF-A_{xxx}b - BS regimen induced VEGF-A_{xxx}b expression, whereas NBS regimen did not (Figure 2C). The same was true for panVEGF-A (Figure 2D) – expression was induced by both regimens, but NBS cells were slower to respond than BS cells. To confirm that this was due to a change in the RNA splice isoforms, we measured RNA levels of VEGF-A_{164}b and VEGF-A_{1654}a by quantitative RT-PCR using isoform specific primers. Figure 2E shows that VEGF-A_{164}a and VEGF-A_{164}b were preferentially upregulated by the NBS and BS regimens, respectively, in BS cells. In NBS cells, the same effect was induced by switching the melatonin regimen, indicating that this effect is specific to the duration of melatonin exposure, rather than the stage of the annual reproductive cycle from which the cell was sourced. These results indicate that melatonin can control angiogenesis protein production in the PT.

VEGF-A splice isoforms and receptors are present in the PD

To determine whether VEGF-A could target endocrine and /or non-endocrine cells that are known to display seasonal plasticity, we screened the PD for the VEGF receptor, VEGFR2. Co-staining of VEGFR2 with folliculostellate cells (FSC, Figure 3A) and lactotrophs (LT, Figure 3B) showed that VEGFR2 was co-localized with a proportion of FSC and LT, and critically, that this co-localization increased ($p<0.01$ and $p<0.001$, respectively) during the non-breeding season (NBS), i.e. in the summer. There was also substantial VEGFR2 expression co-localized on endothelial cells in both seasons (Figure 3C). Screening for

proliferating endothelial cells indicated that there was more angiogenesis in the summer (NBS) in the PD (Figure 3D), as well as the PT and infundibular stalk (Figure 1D). Immunofluorescence staining for VEGF-A isoforms indicated that VEGF-A_{xxx}b was significantly down-regulated and VEGF-A_{xxx}a significantly up-regulated in the summer (NBS, Figure 3E), providing a rationale for the up-regulation of angiogenesis in the PD. Quantification of the area of staining (Figure 3F) confirmed this finding, as did quantitative ELISA for VEGF-A_{xxx}b (down-regulation in the summer –NBS; Figure 3G) and panVEGF-A (Figure 3H – no change – and hence an implied up-regulation of angiogenic isoforms in the NBS).

VEGF-A isoforms control seasonal endocrine function

These results lead to two hypotheses: a) that VEGF-A controls angiogenesis and this allows, by increased portal blood flow, an as yet unidentified compound to repress the reproductive axis (presumably, at least in part, by stimulating prolactin); and/or b) that VEGF-A itself is a signalling molecule from the PT to the PD, which directly contributes to the inhibitory regulation of the reproductive cycle by releasing prolactin. To test this latter hypothesis, we cultured PD cells from sheep in the NBS or BS and treated them with recombinant human VEGF-A_{165}a (rhVEGF-A_{165}a) or conditioned media from the PT cells taken at the same time of year, and measured prolactin production by radioimmunoassay. Figure 4A shows that VEGFR2 and prolactin were both expressed by PD cells in culture. Figure 4B shows that the cells from both NBS and BS animals could be induced to release

prolactin by thyrotropin-releasing hormone (TRH), but not by melatonin. Figure 4C shows that rhVEGF-A_{165}a, given for the duration that matches NBS melatonin exposure (i.e. 8 hrs in the summer), resulted in significant prolactin release from PD cells from NBS animals ($p<0.001$), and from cells from the BS (supplementary Figure 3A). It also showed that rhVEGF-A_{165}a, given at BS duration (16 hours) did not cause prolactin release from BS (Figure 4C) or NBS cells (supplementary Figure 3A). We confirmed this at the RNA level (Figure 4D-E). To determine whether PT cells could generate VEGF-A isoform ratios that induced prolactin, we took conditioned media from the PT cells treated with melatonin and treated the PD cells with this conditioned media to mimic the *in vivo* situation. Conditioned media from PT cells treated with NBS melatonin regimen significantly stimulated prolactin protein (Figure 4F) and mRNA (Figure 4G) in cells from non-breeding season ewes, but did not result in FSH production (supplementary Figure 3B). Critically, this effect was completely blocked by an antibody to VEGF-A_{xxx}a (Figure 4F, G). Conditioned media from PT cells treated with BS melatonin regimen had no effect on prolactin production from breeding season PD cells (Figure 4F), but did stimulate FSH mRNA production from these cells (supplementary Figure 3C), and from cells from non breeding season ewes (supplementary Figure 3D), an effect which was inhibited by pre-treatment with a VEGF-A_{xxx}b specific antibody (supplementary Figure 3D). This indicates that melatonin duration-induced differential VEGF-A isoform production by the PT has the potential to regulate the seasonal production of prolactin and FSH by the PD through an intra-pituitary paracrine mechanism mediated by VEGF.

Figure 2. VEGF-A isoforms levels are regulated by melatonin periodicity in the PT. A. PT cells in culture were isolated from pituitaries of sheep and VEGF-Axxxb measured by ELISA. Cells from winter sheep (breeding season -BS, blue) were treated with melatonin for 16 hrs each day for 6 days; cells from summer sheep (non-breeding season –NBS, red) were treated for 8hrs each day with melatonin. B. Levels of panVEGF-A were also measured from these cells. C. VEGF-Axxxb levels were measured from sheep PT cells incubated with the incongruous melatonin exposure for the time they were harvested from (cells from summer sheep were given winter a melatonin regimen, and from winter sheep given a summer melatonin regimen). D. Levels of panVEGF-A from cells treated as in C. E. VEGF-A isoform mRNA expression in PT cells from the breeding season (BS, winter) after 6 days of treatment with BS and NBS melatonin regimens. F. VEGF-A isoform mRNA expression in PT cells from the non-breeding season (NBS, summer) after 6 days of treatment with BS and NBS melatonin regimens. Boxes show positions of the primers used to amplify the cDNA. ***= $p<0.001$ compared with Control, +++= $p<0.001$ compared with BS Regimen)

Discussion

The rationale for these studies was that dynamic and tightly regulated changes in the vascular communication between the brain and the pituitary gland could underlie seasonal physiology. To that end, we used a highly seasonal animal model, the sheep, with a well-characterized annual reproductive cycle. The results show that the pituitary microvasculature that connects the PT with the neural tissue of the infundibulum, before contacting the PD, displays dramatic seasonal remodelling, and that this could be in response to locally regulated splice variants of VEGF-A in the photoperiodic responsive / melatonin sensitive

PT region. Importantly, we found that the melatonin-induced differential expression of VEGF-A isoforms in the PT throughout the annual reproductive cycle not only has the potential to alter the portal microvasculature, but that itself could operate as a messenger to modify the endocrine output from the PD. We established that the signal from the PT carried to hormone-producing cells in the PD to stimulate prolactin secretion and inhibit FSH during reproductive quiescence is the angiogenic isoform of VEGF, VEGF-A_{164}. Moreover, the results reveal that the splicing of the VEGF-A pre-RNA from anti-angiogenic VEGF-A_{164}b in the winter (BS) to pro-angiogenic VEGF-A_{164}a in the summer (NBS) results from the different duration of melatonin exposure, which occurs across seasons, highlighting the existence of a previously unknown photoperiodically-regulated system for the seasonal control of fertility.

Figure 3. VEGFR2 is upregulated in the PD during the summer (non-breeding season). A. Co-localization of VEGFR2 (green) and glial-type folliculostellate cells (red) in the breeding season (BS, winter) and non-breeding season (NBS, summer). B. VEGFR2 (green) expression in lactotrophs (red) in the BS and NBS. C. VEGFR2 (green) and endothelial cells stained by isolectinB4 (IB4, red) in both seasons. D. Proliferating (PCNS, green) endothelial (IB4, red) cells were stained, and co-localization quantified. E. VEGF-Axxxb expression detected in the PD in the winter (BS), but not in the summer (NBS); VEGF-Axxx expression detected in the summer (NBS) but not in the winter (BS). F. Quantification of the expression of VEGF-A isoforms. G. ELISA quantification of the amount of VEGF-Axxxb in the BS and NBS. H. ELISA quantification of the amount of total VEGF-A in the two seasons. *= $p<0.05$, **= $p<0.01$, and ns= non-significant ($p>0.05$) compared with BS. Scale bar = 50µm

The microvasculature of the pituitary gland is key to the regulation of multiple body functions because it controls the blood flow from the hypothalamus, altering the delivery of stimulatory and inhibitory signals to endocrine target cells [20]. Here we show that this vascular connection undergoes a remarkable seasonal adaptation throughout the annual reproductive cycle in response to an external cue, namely the changing photoperiod. Such vascular plasticity is manifested in alterations of endothelial cell proliferation that result in timely changes in the vascular loops that connect the PT with the infundibulum before giving rise to the long portal vessels which terminate in the PD. Even though these loops were first described over six decades ago [21], the possibility that they could alter the connectivity between the brain and the pituitary at certain times of the year was not known. Although no apparent alterations in the ovine pituitary vasculature of male castrates exposed to different photoperiods was recently reported, detailed measurements were not undertaken and the vascular loops were not specifically examined [22]. The increased number and surface area of these vascular loops during the long days of the NBS, concomitant with an increase in endothelial cell proliferation, is in agreement with the increased number of cells proliferating in the PT shown in this study and by another group [23] at this time of year.

Figure 4. VEGF-A mediates prolactin release from the PD in an isoform dependent manner. A. VEGFR2 expression in cultured prolactin positive cells (lactotrophs) of the PD. B. Prolactin secretion following treatment with thyrotropin-releasing hormone (TRH, positive control), melatonin (Mel, negative control) and medium (control) in PD cells cultured during the non-breeding season (NBS, summer) and breeding season (BS, winter). C. Prolactin secretion in PD

cells from sheep killed in the summer (NBS) after rhVEGF-A165a treatment was greater than that from winter (BS) sheep. D. Prolactin mRNA expression in PD cells taken from ewes in the summer (NBS) following a summer (NBS) regimen of rhVEGF-A165a (8hrs on, 16hrs off), was greater than that of cells taken from the same animals following a winter (BS) rhVEGF-A165a regimen (16hrs on, 8hrs off). E In cells taken from ewes in the breeding season (BS, winter), prolactin was only induced if VEGF-A was given in a non-breeding season (NBS, summer) regimen, F. Conditioned media from summer (NBS) PT cells treated with a summer (NBS) melatonin regimen (red) induced prolactin production. This was blocked by an antibody to VEGF-Axxxa. G. Prolactin mRNA in summer (NBS) cells was induced in the presence of conditioned media from summer (NBS) regimen PT cells, and this was blocked by an anti-VEGF-Axxxa antibody. **=p<0.01, ***=p<0.001 compared with BS. Scale bar = 20μm

The afferent branches of these loops connect the photoperiodic-responsive PT with the FS cell-rich infundibulum, and efferent branches provide communication between the infundibulum and the PD where most of the pituitary hormones are produced. Thus, the temporal remodelling of the vascular connection between these three tissues highlights the existence of a control point for seasonal physiology. The importance of this finding is supported by recent studies in rodents showing that the pituitary microvasculature adapts to the needs of pituitary endocrine cells and that it can control endocrine output according to the physiological requirement of the individual [24]. This remodelling could substantially increase or decrease the transport of hormones from the PT to the infundibulum and from the infundibulum to the PD, thus being able to accentuate or reduce the effect of hypothalamic derived neuroendocrine signals, such as GnRH, to the hormone producing cells of the pituitary. It is possible that this

regulatory mechanism for the delivery of hypothalamic factors could operate in conjunction with the dynamic retraction / protraction of ‘endfeet’ processes of tanycytes [25], the specialized ependymal cells of the glia, which have been shown to interact with the hypothalamic neuronal terminals and fenestrated capillaries of the median eminence and to play a role in the control of the seasonal reproductive cycle in birds [26]. The VEGF isoforms have been shown to differentially regulate fenestrations of endothelial cells [16], so it is possible that the two mechansims could work together, although we are unable to use the evidence presented here to differntiate between dependnet and independent mechanisms.

In other tissues, vascular remodelling and permeability is controlled by VEGF-A [19], so dynamic changes in the pituitary microvasculature were expected to correspond to alterations in VEGF-A expression. Indeed, VEGF-A-mediated changes in endothelial cell proliferation and angiogenesis were communicated in specific regions of the songbird brain across seasons [27]. However, VEGF-A had been previously reported to remain unchanged in the pituitary of sheep under different photoperiods [28], and in this study panVEGF-A expression did not differ between BS and NBS animals. Critically, the use of specific antibodies to the pro- and anti-angiogenic isoforms of VEGF-A (these distinguish between isoform families, but not between the different length isoforms, hence VEGF-A_{xxx}a and VEGF-A_{xxx}b) revealed differential isoform expression between the long days of the NBS and the short days of the BS, with over-expression of anti-

angiogenic VEGF-A_{xxx}b variants in the BS, and increased expression of pro-angiogenic VEGF-A_{xxx}a variants in the NBS, providing an explanation for the observed changes in the microvasculature. As the PT is the tissue with the highest density of melatonin receptors [4, 5], and reliably translates the effects of photoperiod on circadian and circannual physiology within the pituitary [1, 29], our results showing the co-expression of VEGF-A and melatonin receptors in PT-specific cells provide compelling evidence that the seasonal regulation of the vascular connection between the brain and the pituitary gland is mediated by a melatonin-induced mechanism within the PT region that leads to differential expression of pro- and anti-angiogenic VEGF-A isoforms. Moreover, our results show that in addition to the PT-specific cells, melatonin could also act directly on the vascular loops, revealing a previously unknown target for melatonin action to translate photoperiodic effects on seasonal physiology. As the blood flows from the brain to the pituitary [11], alterations in the vascular loops of the infundibulum that will give rise to the long portal vessels [12] could contribute not only to regulate the transfer of PT products to the PD, but also to alter the delivery of hypothalamic factors; thus, the increased vascular connections during the long days of summer would be expected to favor increased supply of stimulatory and inhibitory hypothalamic signals to the PD at this time of year. Notwithstanding, the reduction in vascularity during the short days of winter is likely to play a role in the modulation of the gonadotroph response to GnRH, by means of preventing desensitization of GnRH receptors [30], and fine-tuning the differential control of

gonadotropin secretion [31, 32], which are essential processes to ensure normal fertility.

Photoperiodic information is encoded by the duration of nocturnal melatonin secretion [2], so we employed a paradigm where ovine PT cells were cultured and daily exposed to summer (NBS) or winter (BS) durations of melatonin treatments (8 vs. 16h, respectively) over a period of six days. PT cells from the same animals were exposed to both the matching and non-matching (i.e. opposite season) melatonin regimens, so we were able to differentiate direct effects of the melatonin signal and those resulting from its interaction with the circannual phase. We show that duration of melatonin exposure induced a striking differential expression of VEGF-A isoforms, with up-regulation of the pro-angiogenic isoform VEGF-A_{xxx}a by a short duration (i.e. 8h, summer; NBS) regimen, and up-regulation of the anti-angiogenic isoform VEGF-A_{xxx}b by a long duration (i.e. 16h, winter; BS) regimen. This melatonin duration-dependent differential expression of VEGF-A isoforms was also recorded in cells obtained in the opposite season but at a slower rate, highlighting the requirement of PT cells to be entrained to the new signal. Thus, the results are consistent with the findings ex-vivo and demonstrate that pituitary micro-vascular remodelling is likely to be sensitive to the changing photoperiod and adapts to the physiological requirements of the animal in response to time-dependent melatonin signals acting on VEGF-A. The mechanism through which melatonin switches splicing of the VEGF-A gene is not yet known, but alternative splicing of VEGF has been

shown to be regulated by activation of the RNA binding proteins SRSF1, SRSF2 and SRSF6 by the kinases SRPK1 and Clk4 [33].

We then investigated whether the seasonal regulation of VEGF-A in the PT could affect the function of the PD. We show that VEGF-A receptors are expressed in endocrine, endothelial and FS cells in the PD and that their co-localization is also under seasonal control, with up-regulation during the long days of the NBS. In addition, there was increased content of pro-angiogenic VEGF-A isoforms at this time of year and, conversely, increased content of the anti-angiogenic isoforms during the short days of the BS. Since the seasonal regulation of VEGF-A isoform expression was shown to be melatonin-dependent and, in accordance with previous studies [34], the PD was shown not to contain melatonin receptors, the varying content of VEGF-A isoforms in the PD is likely to rely on a paracrine mechanism [35]. The physiological significance of this was first revealed in the PD microvasculature, with increased endothelial cell proliferation demonstrated during the NBS. We show that this increase in angiogenesis at this time of the annual reproductive cycle is concomitant with an increase in the prevalence of FS cells containing VEGF receptors. FS cells are glial-like, non-endocrine cells that, via gap-junctions, generate a three-dimensional network throughout the pituitary to coordinate its function [36, 37]. These cells secrete an array of paracrine factors known to influence endocrine cells such as gonadotrophs and lactotrophs, and are a primary source of VEGF-A [35]. In seasonal breeders, FS cells are distributed throughout the PD and PT [38] and respond to photoperiodic

changes with a high degree of plasticity [39, 40]. In sheep, significant ultra-structural changes together with enhanced number of intercellular adherens junctions and increased number of elongated processes surrounding endocrine cell clusters were reported during the long days of the NBS [41]. As FS cells do not contain melatonin receptors [42], our findings revealing up-regulation of VEGF receptor content in these cells at this time of year provide evidence for a role of VEGF-A in the dynamic changes of the FS cell network to control vascular plasticity via the regulation of its own production during the annual reproductive cycle. The seasonally regulated differential expression of VEGF-A isoforms in the pituitary gland of a short day breeder unravelled here, could also operate in long day breeders, such as hamsters and horses, as part of the mechanisms controlling their annual physiology. Indeed, preliminary results have provided evidence that, in Thoroughbred horses, VEGF-A isoform expression in both, the PT and PD regions of the pituitary, is also seasonally regulated [43], suggesting that this is a conserved mechanism for seasonal adaptation in photoperiodic mammals.

Notably, we show that, in addition to its actions on the pituitary vasculature and FS cell population, VEGF-A has a potent prolactin releasing effect, and that this stimulation depends on time of exposure of the ligand and density of VEGF receptors in lactotrophs, which is increased during the long days of the NBS. Moreover, these stimulatory effects of VEGF-A on prolactin synthesis and release were accompanied by suppression of the gonadotrophic axis, as

revealed by inhibition of FSH gene expression. Melatonin was shown to mediate the photoperiodic regulation of prolactin secretion through a direct action within the pituitary gland [6]. Because MT1 melatonin receptors are selectively expressed in the PT, and this region is deprived of lactotroph cells [7, 8], a paracrine mechanism for the control of prolactin secretion from the PD is warranted. Activation of MT1 melatonin receptors in the PT is known to inhibit adenylyl cyclase, and pharmacological studies in sheep have shown that melatonin impairs forskolin-induced hypersecretion of cyclic adenosine monophosphate (cAMP), with inhibition of prolactin from the PD through the reduction of a paracrine signal [44]. However, although several compounds such as tachykinins, substance P and neurokinin A are produced by the PT and can stimulate prolactin release [45-47], characterization of the chemical identity of that signal has been elusive. Here we show that the stimulatory effects of VEGF-A on prolactin were mimicked by conditioned media from PT cultures exposed to a NBS regimen of melatonin, and that these actions of PT media were blocked by a specific VEGF-A_{xxx}a antibody, demonstrating that VEGF-A is a potential paracrine signal, and that melatonin-induced differential VEGF-A isoform production by the PT can regulate the seasonal production of both prolactin and FSH. Because these effects were also recorded in PD cells obtained in the opposite season (i.e. BS), albeit with a three-day lag required for adaptation, our results show that the photoperiodically-induced paracrine mechanism mediated by VEGF-A can ultimately override the circannual phase of the PD target cells, and entrain it to the new photoperiod. The increased VEGF receptor content in

the PD during the NBS plays a major role in mediating this process, and thus in the biological adaptation to a summer physiology, because VEGF-A treatments mimicking a NBS melatonin regimen showed a delayed response in BS cultures where the VEGF receptor content was reduced.

Figure 5. Working model for a melatonin-induced, VEGF-A isoform dependent intra-pituitary regulation of seasonal physiology. In this model, the duration of nocturnal melatonin secretion induces differential synthesis and release of pro-angiogenic and anti-angiogenic isoforms of VEGF-A in the PT region of the ovine pituitary and in the vascular loops that connect this tissue with the infundibulum. A. In the short days of winter (BS), the long duration of nocturnal melatonin exposure up-regulates the secretion of the anti-angiogenic isoform VEGF-A164b at the expense of the pro-angiogenic isoform VEGF-A164a, resulting in reduced angiogenesis, reduced density of VEGF receptors in endocrine and FS cells of the PD, suppression of prolactin secretion and no inhibition of the gonadotrophic axis. B. In contrast, during the long days of summer (NBS), the short duration of nocturnal melatonin exposure up-regulates the secretion of the pro-angiogenic isoform VEGF-A164a at the expense of the anti-angiogenic isoform VEGF-A164b, leading to increased angiogenesis, increased density of VEGF receptors in endocrine and FS cells of the PD, stimulation of prolactin secretion and inhibition of the gonadotrophic axis.

Entrainment of the PD cells to a specific phase of the circannual cycle explains why NBS cells failed to secrete prolactin in response to the first 8 hours of either a BS (16 h) VEGF-A regimen, or PT conditioned media from the BS.

In rodents, melatonin-induced suppression of cAMP is followed by sensitization of adenosine A_{2b} receptor signalling, leading to subsequent increase in cAMP and CREB phosphorylation [28]. Disruption of this signalling pathway in MT1

melatonin receptor knock out mice resulted in altered prolactin secretion, implicating cAMP and adenosine in this biological response to melatonin. Our results indicate that VEGF-A is likely to be downstream of that pathway to bring about the biological response. Indeed, cAMP signalling, CREB phosphorylation and adenosine are associated with angiogenesis [48, 49] via stimulation of VEGF-A [50], and whereas pharmacologically induced cAMP up-regulation and treatment with adenosine stimulated VEGF-A expression in smooth muscle cells [50], the selective knockdown of all VEGF-A isoforms blocked the actions of elevated cAMP on hippocampal neurons [51]. The melatonin induced VEGF-A regulation of prolactin secretion shown in this study will have an impact on the gonadotrophic axis in addition to its direct inhibition of FSH, because when combined with dopamine prolactin impairs the gonadotroph response to GnRH in a seasonal dependent manner in both long and short day breeders [52-54].

Our results provide evidence for an intra-pituitary mechanism that responds to an external independent signal to regulate seasonal physiology. We propose a model where the duration of nocturnal melatonin secretion promotes alternative splicing of the VEGF-A gene leading to differential synthesis and release of pro-angiogenic and anti-angiogenic isoforms of VEGF-A within the PT region of the pituitary gland and in the vascular loops that connect this tissue with the infundibulum (Figure 5). The resulting output of VEGF-A isoforms will have two complementary effects: 1) it alters the temporal vascular connection between the brain and the pituitary gland; and 2) it can be used as a paracrine signal to

modify the seasonal activity of endocrine cells in the PD that control reproduction. In this model, the long duration of nocturnal melatonin exposure during the winter up-regulates the secretion of anti-angiogenic isoforms VEGF-A_{xxx}b at the expense of pro-angiogenic isoforms VEGF-A_{xxx}a, resulting in reduced angiogenesis, reduced density of VEGF receptors in endocrine and FS cells, suppression of prolactin secretion and no inhibition of the gonadotrophic axis characteristic of the BS. Conversely, the short duration of nocturnal melatonin exposure during the summer will up-regulate the secretion of pro-angiogenic isoforms VEGF-A_{xxx}a at the expense of anti-angiogenic isoforms VEGF-A_{xxx}b, leading to increased angiogenesis, increased density of VEGF receptors in endocrine and FS cells, stimulation of prolactin secretion and inhibition of the gonadotrophic axis, characteristic of the NBS. Thus, the model permits a physiological adaptation to the seasonal requirements of the species by means of an angiogenesis dependent intercommunication between two regions of the pituitary.

Materials and Methods

Details of standard protocols are given in the supplementary materials. Ovine pituitary glands were obtained from ovary-intact females during the breeding season (BS -December⁄January) and the non-breeding season (NBS -June ⁄July). Animals were killed for commercial reasons at an abattoir and pituitaries removed immediately after death. During the BS, ewes were confirmed to be sexually active on the basis of a recently formed corpus luteum (CL) together

with the presence of a large follicle (> 2 cm). By contrast, in the NBS, ewes were considered to be anestrus when no CL but a corpus albicans was observed in the gonad, and follicles present were < 2 mm diameter.

Expression studies

Pituitaries were stained and RNA was extracted [55] using standard procedures (details of antibodies in supplementary Tables 1-2 and primers shown in supplementary Table 3). The term VEGF-A_{xxx}b is used as the antibodies do not distinguish between the different VEGF-A_{xxx}b isoforms (e.g. VEGF-A_{121}b, VEGF-A_{165}b, VEGF-A_{189}b). The term VEGF-A_{164}b or VEGF-A_{165}b is used when the methodology specifically describes the sheep 164 amino acid isoform (isoform specific RT-PCR, as the forward primers cross Exon 5 and Exon 7), or the human 165 amino acid isoform when recombinant protein is used).

Primary cell cultures

Ovine primary pituitary cultures were produced by careful dissection and dissociation of the pars distalis (PD) and pars tuberalis (PT) of 3-4 pituitaries as previously described 52. Previous studies have demonstrated the validity of this method for producing a reliable hormone output in response to exogenous hormone releasing secretagogues in vitro [52, 56].

Both ELISA methods have been previously described [57-58,11]. A rhVEGF$_{165}$b positive control was included in triplicate for the human VEGF-A ELISA, allowing

calculation of VEGF-A_{total} concentration to compensate for reduced VEGF-A_{xxx}b affinity, approximately 42%, as previously published [59]. Prolactin was measured by RIA using purified ovine prolactin for standards. A linear relationship was detected when the measured hormone concentration (ng／ml) was plotted against the concentration of diluted serum samples.

Statistical analysis

In both the BS and NBS cultures, a total of five separate experimental treatments were applied to PT cells, and nine experimental treatments applied to the PD cells. For each treatment, six wells were assigned, and the experiments were repeated independently three times in both seasons with reproducible results. The reported values represent the mean +/-SEM. The effects of season, experimental treatment and their interaction on the secretion VEGF-A and prolactin from ovine primary pituitary cell cultures were examined using ANOVA followed by Fisher's post-hoc test. Because a season by treatment interaction was observed for each compound, separate ANOVAS were then used to examine the effects of experimental treatment within season. For all other variables one-way ANOVA was applied. All data were confirmed to be normally distributed by D'Agostino and Pearson omnibus normality test. Data were considered to be statistically significant when $p<0.05$; however, wherever detected, smaller log value ($p<0.01$, $p<0.001$) probabilities are reported.

Author contributions

Experimental work was carried out by JCM and analyzed by JCM, DT and DOB. The work was conceived, designed and supervised DOB and DT. The manuscript was written by DOB, DT and JCM.

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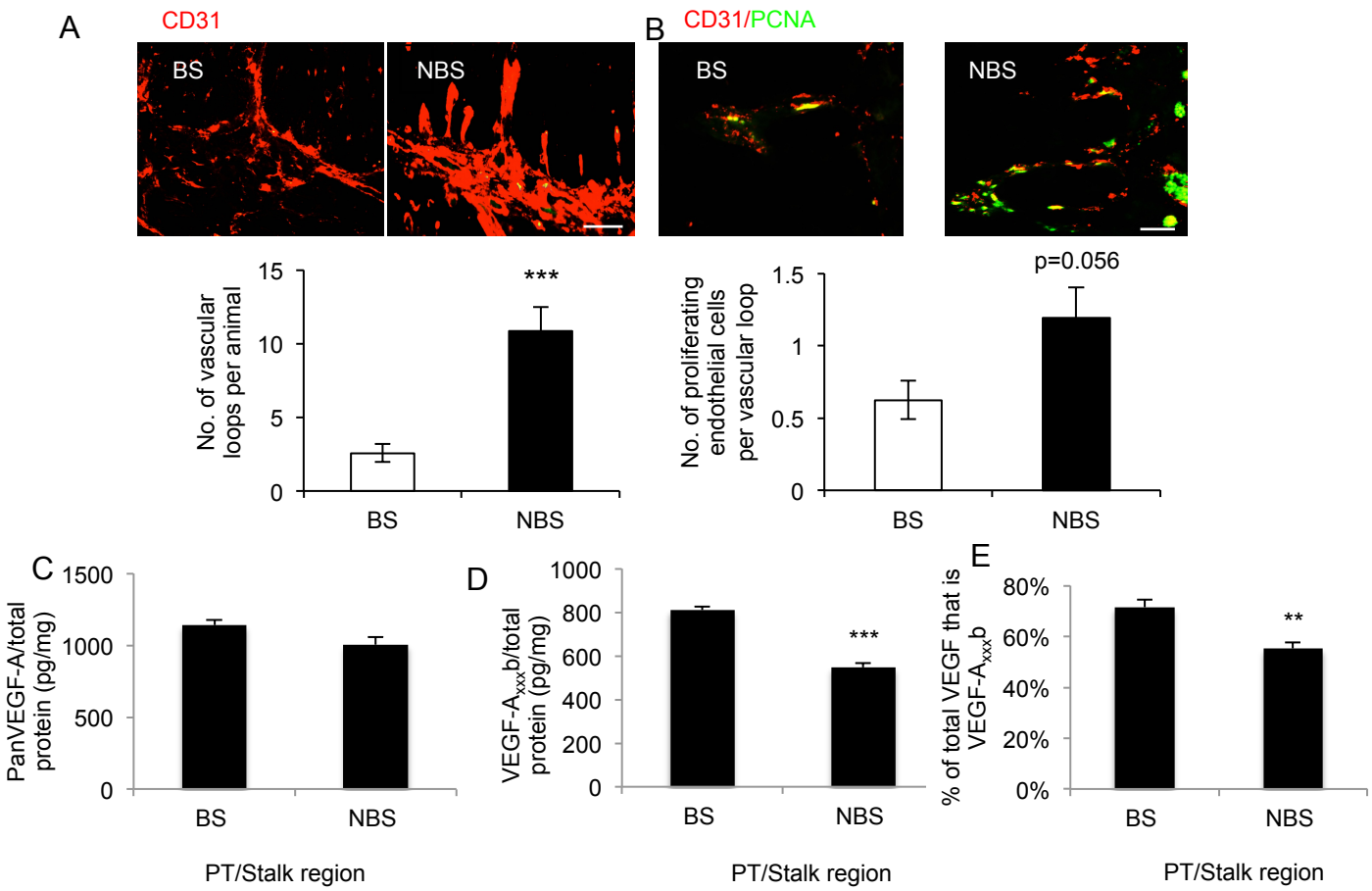
A
CD31
BS
NBS
B
CD31/PCNA
BS
NBS
No. of vascular loops per animal
15
10
5
0
***
BS
NBS
No. of proliferating endothelial cells per vascular loop
1.5
1
0.5
0
p=0.056
BS
NBS
C
PanVEGF-A/total protein (pg/mg)
1500
1000
500
0
BS
NBS
PT/Stalk region
D
VEGF-Axxxb/total protein (pg/mg)
1000
800
600
400
200
0
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BS
NBS
PT/Stalk region
E
% of total VEGF that is VEGF-Axxxb
80%
60%
40%
20%
0%
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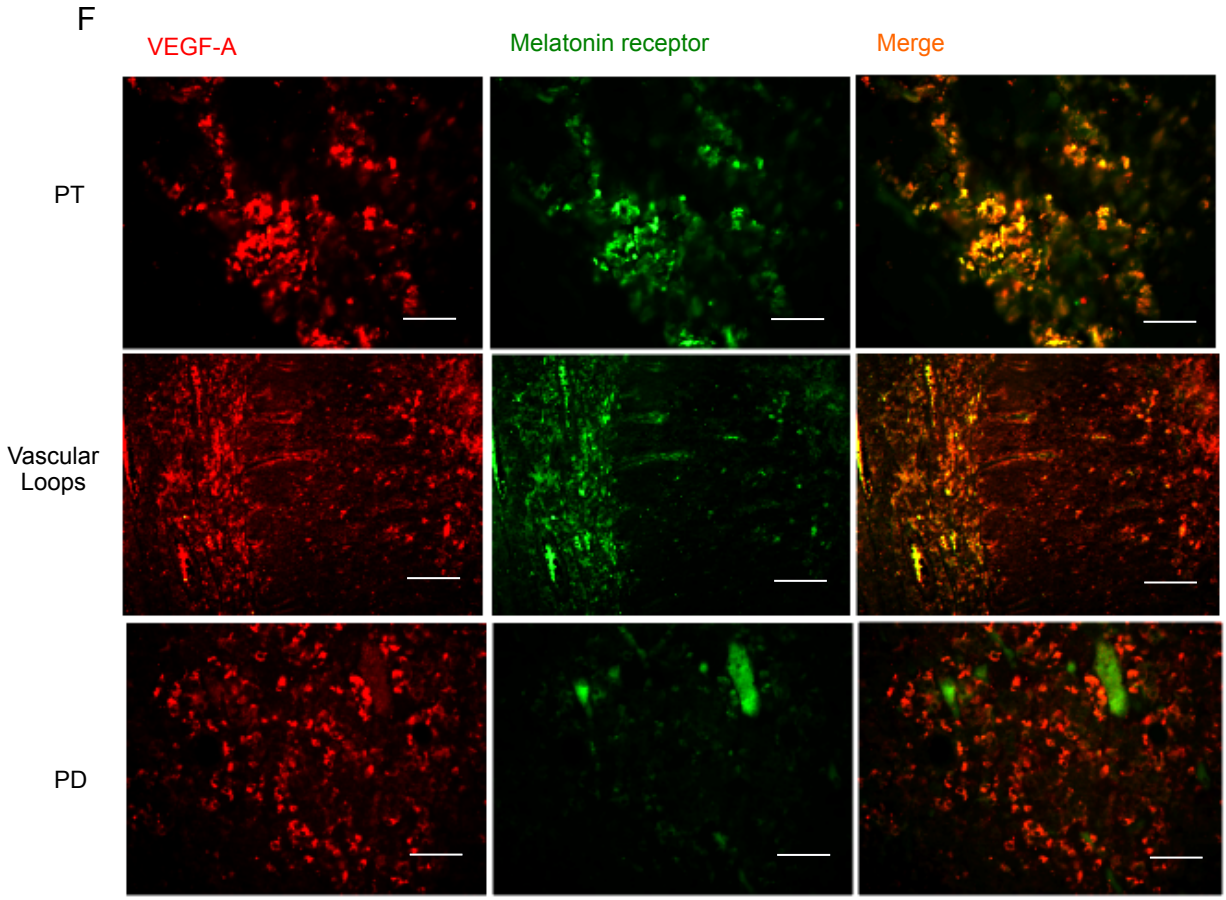
BS
NBS
PT/Stalk region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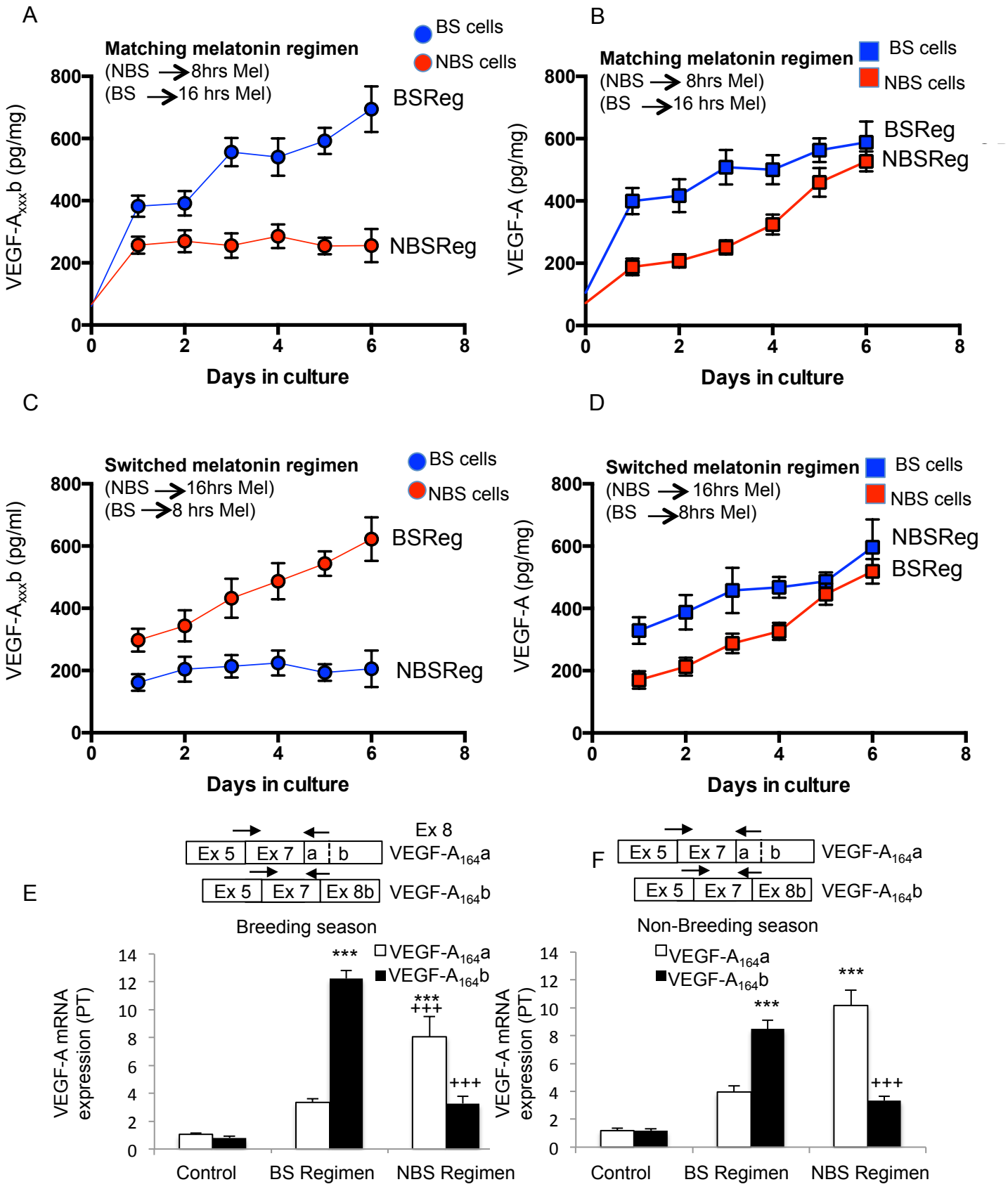
VEGF-A
Melatonin receptor
Merge
PT
Vascular Loops
PD


A
Matching melatonin regimen
(NBS → 8hrs Mel)
(BS → 16 hrs Mel)
BS cells
NBS cells
BSReg
NBSReg
VEGF-Axxxb (pg/mg)
Days in culture
B
Matching melatonin regimen
(NBS → 8hrs Mel)
(BS → 16 hrs Mel)
BS cells
NBS cells
BSReg
NBSReg
VEGF-A (pg/mg)
Days in culture
C
Switched melatonin regimen
(NBS → 16hrs Mel)
(BS → 8 hrs Mel)
BS cells
NBS cells
BSReg
NBSReg
VEGF-Axxxb (pg/ml)
Days in culture
D
Switched melatonin regimen
(NBS → 16hrs Mel)
(BS → 8hrs Mel)
BS cells
NBS cells
NBSReg
BSReg
VEGF-A (pg/mg)
Days in culture
E
Ex 5
Ex 7
a
b
Ex 8
VEGF-A164a
Ex 5
Ex 7
Ex 8b
VEGF-A164b
Breeding season
VEGF-A mRNA expression (PT)
VEGF-A164a
VEGF-A164b
***
***
+++
+++
Control
BS Regimen
NBS Regimen
F
Ex 5
Ex 7
a
b
VEGF-A164a
Ex 5
Ex 7
Ex 8b
VEGF-A164b
Non-Breeding season
VEGF-A mRNA expression (PT)
VEGF-A164a
VEGF-A164b
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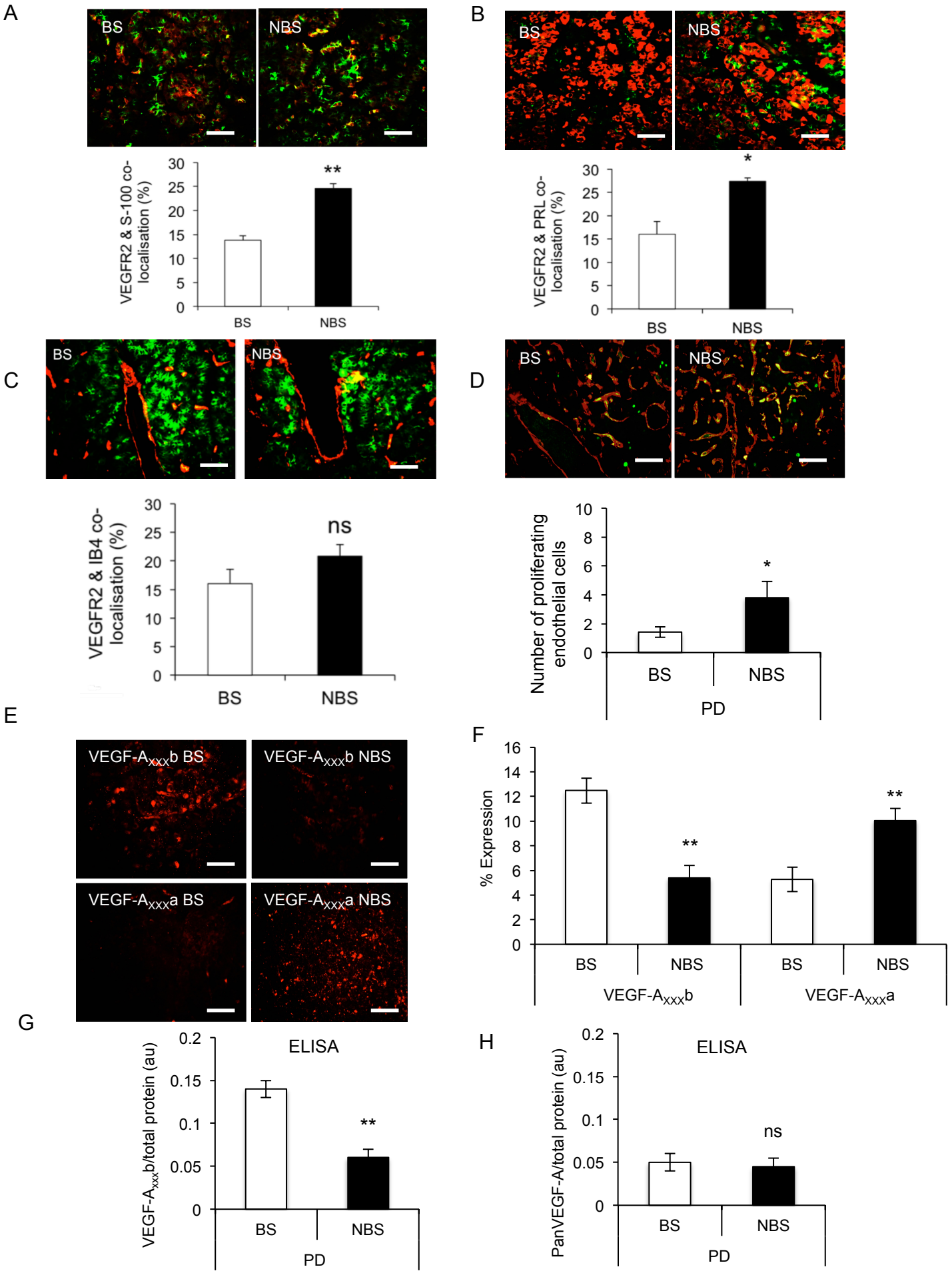
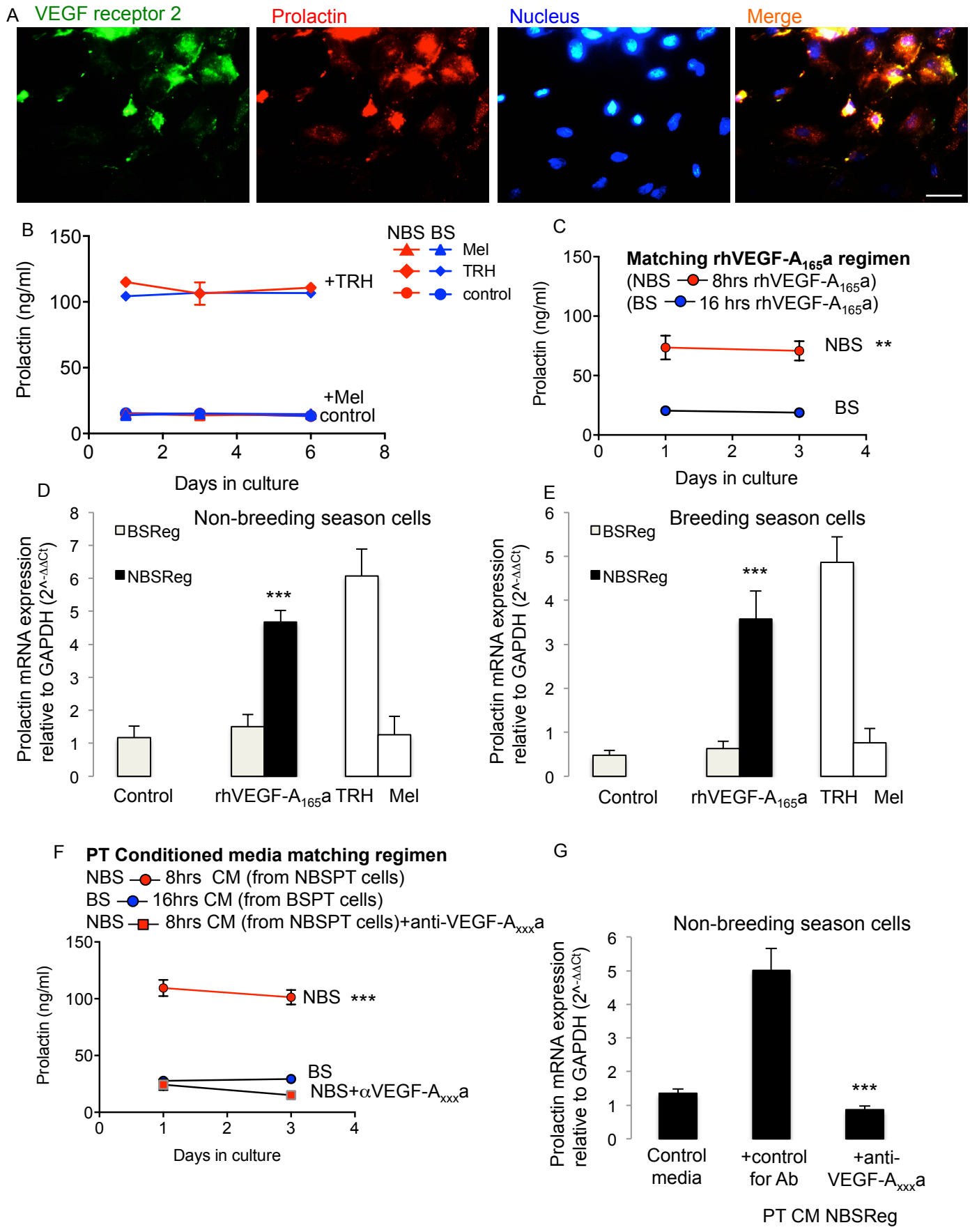
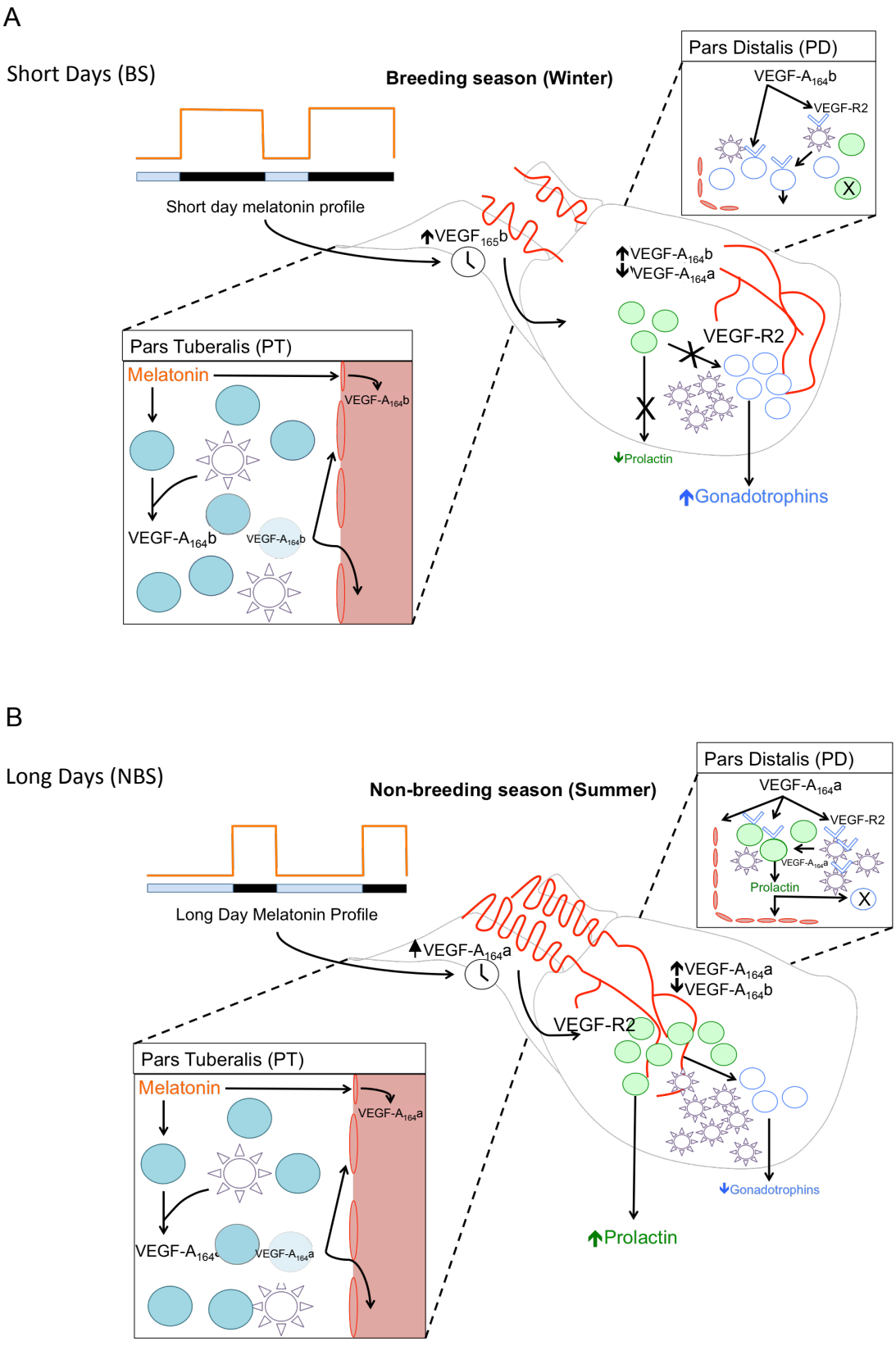
***
+++
Control
BS Regimen
NBS Regimen


A
BS
NBS
VEGFR2 & S-100 co-localisation (%)
30
25
20
15
10
5
0
**
BS
NBS
B
BS
NBS
VEGFR2 & PRL co-localisation (%)
30
25
20
15
10
5
0
*
BS
NBS
C
BS
NBS
VEGFR2 & IB4 co-localisation (%)
30
25
20
15
10
5
0
ns
BS
NBS
D
BS
NBS
Number of proliferating endothelial cells
10
8
6
4
2
0
*
BS
NBS
PD
E
VEGF-A$_{xxx}$b BS
VEGF-A$_{xxx}$b NBS
VEGF-A$_{xxx}$a BS
VEGF-A$_{xxx}$a NBS
F
% Expression
16
14
12
10
8
6
4
2
0
**
**
BS
NBS
BS
NBS
VEGF-A$_{xxx}$b
VEGF-A$_{xxx}$a
G
ELISA
VEGF-A$_{xxx}$b/total protein (au)
0.2
0.15
0.1
0.05
0
**
BS
NBS
PD
H
ELISA
PanVEGF-A/total protein (au)
0.2
0.15
0.1
0.05
0
ns
BS
NBS
PD


A
VEGF receptor 2
Prolactin
Nucleus
Merge
B
Prolactin (ng/ml)
NBS BS
Mel
TRH
control
+TRH
+Mel
control
Days in culture
C
Matching rhVEGF-A165a regimen
(NBS 8hrs rhVEGF-A165a)
(BS 16 hrs rhVEGF-A165a)
NBS **
BS
Prolactin (ng/ml)
Days in culture
D
Non-breeding season cells
BSReg
NBSReg
***
Prolactin mRNA expression relative to GAPDH (2^-ΔΔCt)
Control
rhVEGF-A165a
TRH
Mel
E
Breeding season cells
BSReg
NBSReg
***
Prolactin mRNA expression relative to GAPDH (2^-ΔΔCt)
Control
rhVEGF-A165a
TRH
Mel
F
PT Conditioned media matching regimen
NBS 8hrs CM (from NBSPT cells)
BS 16hrs CM (from BSPT cells)
NBS 8hrs CM (from NBSPT cells)+anti-VEGF-Axxxa
NBS ***
BS
NBS+αVEGF-Axxxa
Prolactin (ng/ml)
Days in culture
G
Non-breeding season cells
Prolactin mRNA expression relative to GAPDH (2^-ΔΔCt)
***
Control media
+control for Ab
+anti-VEGF-Axxxa
PT CM NBSReg

Intra-pituitary control of seasonal breeding



Supporting Information

S1 Materials and Methods

EXPERIMENTAL PROCEDURES

Ovine pituitary glands were obtained from ovary-intact females during the breeding season (BS -December⁄January) and the non-breeding season (NBS - June⁄July). Animals were killed for commercial reasons at an abattoir (University of Bristol Abattoir, Langford, UK) and pituitaries removed immediately after death. During the BS, ewes were confirmed to be sexually active on the basis of a recently formed corpus luteum (CL) together with the presence of a large follicle (> 2 cm). By contrast, in the NBS, ewes were considered to be anestrus when no CL but a corpus albicans was observed in the gonad, and follicles present were < 2 mm diameter.

Immuno-fluorescent staining

Pituitaries assigned for immunofluorescent staining (BS n = 6; NBS n =6) were fixed in Bouin's solution for 24 hrs and then moved to 70% ethanol, and sectioned at 5μm. Following sequential dehydration, sections were submerged in PBS-T (0.1% Triton-X) and then 0.01M sodium citrate buffer (pH6; Sigma) and heated for 3 min at full power, and 12 min at sub-boiling temperature. Sections were then washed in PBS-T (3 x 5 min), and blocked in 5% goat serum diluted in 1% BSA PBS-T (0.01%) for 2 hrs at RT. A range of primary antibodies were used for double florescent immunohistochemistry (IMF), each diluted to a concentration determined during preliminary investigations (Table 1). Secondary antibodies were diluted as outlined in Table 2 and left to incubate on the section for 2 hrs at RT.

cDNA synthesis and RT-PCR and RT-qPCR

Pituitaries assigned for DNA analysis of VEGF-A expression were flash-frozen in liquid nitrogen following dissection. RNA Extraction was carried out by TRI reagent method, itself a modification from the original phenol/chloroform extraction developed by Chomczynski et al. [60]. Multiple pairs of primers were used to amplify the various VEGF-A isoforms (Table 3). To generate cDNA, 2μg of RNA and 2 units of RNase-Free DNase (RQ1, M6101, Promega) were incubated in a 1x reaction buffer solution for 1 hour at 37°C, before 1μl of DNase stop solution was added to terminate the reaction, and the sample was heat inactivated for 10 minutes at 65°C. The DNase-treated RNA sample was re-quantified by using a Nanodrop ND-1000 spectrophotometer and 1μg of RNA was re-suspended to a total volume of 10μl. To this, 2μl of Oligo $(dT)_{15}$ primers (C1101, Promega) and 1μl of hexamers (Random Primers, C1181, Promega) were added. The reaction mix was then incubated at 70°C for 10 minutes before immediately being quenched on ice for 5 minutes. With a final reverse

transcription reaction volume of 50µl, 400 units of MMLV reverse transcriptase (M5301, Promega), 40 units of RNasin ribonuclease inhibitor (N2611, Promega) and 0.5mM dNTPs (BIO-39049, Bioline) were added to the RNA/primer mix. The reaction was incubated for 1.5 hours at 37°C with a final 70°C inactivation step. Final concentration of fresh cDNA was determined by spectrophotometry.

For RT-PCR, forward and reverse primer (1µM, see Table 3) were added each with 1.2mM MgCl2, 200µM deoxynucletide triphosphates, and 1 unit of Taq polymerase (Abgene, Thermo-Fischer, Epsom, UK). PCR was undertaken for 35 cycles, at 95°C for 1 min, 60°C for 5 mins and 72°C for 5 mins with a 2 minute 95°C denaturing step at the beginning and a 72°C extension step at the end. PCR products were run on 3% agarose gels containing 0.5 µg/ml Ethidium Bromide and visualized under a UV transilluminator. For RT-qPCR, cDNA was added to 5.5µl of SYBRG fast track with Rox (Kappa Biosystems, Wilmington, Massachusetts, USA), 1µM of each primer in a total of 18µl. Samples were loaded in triplicate and a negative control of water was added. GAPDH was used as a reference gene.

Primary cell cultures

Ovine primary pituitary cultures were produced using a method previously described [61]. Briefly, the pars distalis (PD) and pars tuberalis (PT) of 3-4 pituitaries were carefully dissected and incubated in a 0.1% collagenase D (Boehringer Mannheim, Mannheim, Germany) and hyaluronidase (Sigma-Aldrich, Poole, UK) solution in a shaking water bath at 37 °C for 75 min. The tissue was then manually dispersed in phosphate-buffered saline (Sigma-Aldrich) and the mixed pituitary cells re-suspended in M199 medium (Invitrogen, Paisley, UK) containing 10 mg/ml of insulin, 50 mg/ml of gentamicin, 100 IU/ml of penicillin-streptomycin (Sigma-Aldrich) and 10% steroid-free lamb serum (Invitrogen), before being plated at a density of 200 000 cells/well in 24-well plates. The experiment was repeated three times each in the BS and NBS, totalling 9-12 animals per season. During both the BS and the NBS, cells were maintained in culture for 6 days. M199 media was changed at each time point outlined below. Previous studies have demonstrated the validity of this method for producing a reliable hormone output in response to exogenous hormone releasing secretagogues in vitro [61, 62].

PT cells were assigned one of the following treatments; i) Control - M199 media alone, changed daily at 5pm and 9am; ii) BS regimen – melatonin (1µM), administered at 5pm, removed at 9am, media alone administered from 9am until 5pm; iii) NBS regimen - melatonin administered at 9pm, removed at 5am, media from 5am until 9pm. Three experimental groups of PT cells per season were used. Six wells were assigned per treatment, 3 wells used for RNA based assays, and 3 wells for protein based assays. In one repeat of each experiment per season, 1 well per treatment was used for IMF. PD cells were treated with the following treatments: i) Control (C) – M199 media alone, changed every day at 5pm and 9am; ii) BS regimen - recombinant human VEGF-A_{165}a (1nM rh.VEGF-

$A_{165}a$), or 1µM melatonin, or 0.1µM thyrotropin releasing hormone (TRH), or conditioned media from PT cells treated as above administered at 5pm, removed at 9am, and media alone administered from 9am until 5pm; iii) NBS regimen rh.VEGF-$A_{165}a$ melatonin, or TRH, or conditioned media from PT cells treated as above, administered at 9pm, removed at 5am, media alone administered from 5am until 9pm. Rh.VEGF-$A_{165}a$ was administered at 1nM based on preliminary investigations that produced a VEGF-A dose response to melatonin from PT primary cells in culture.

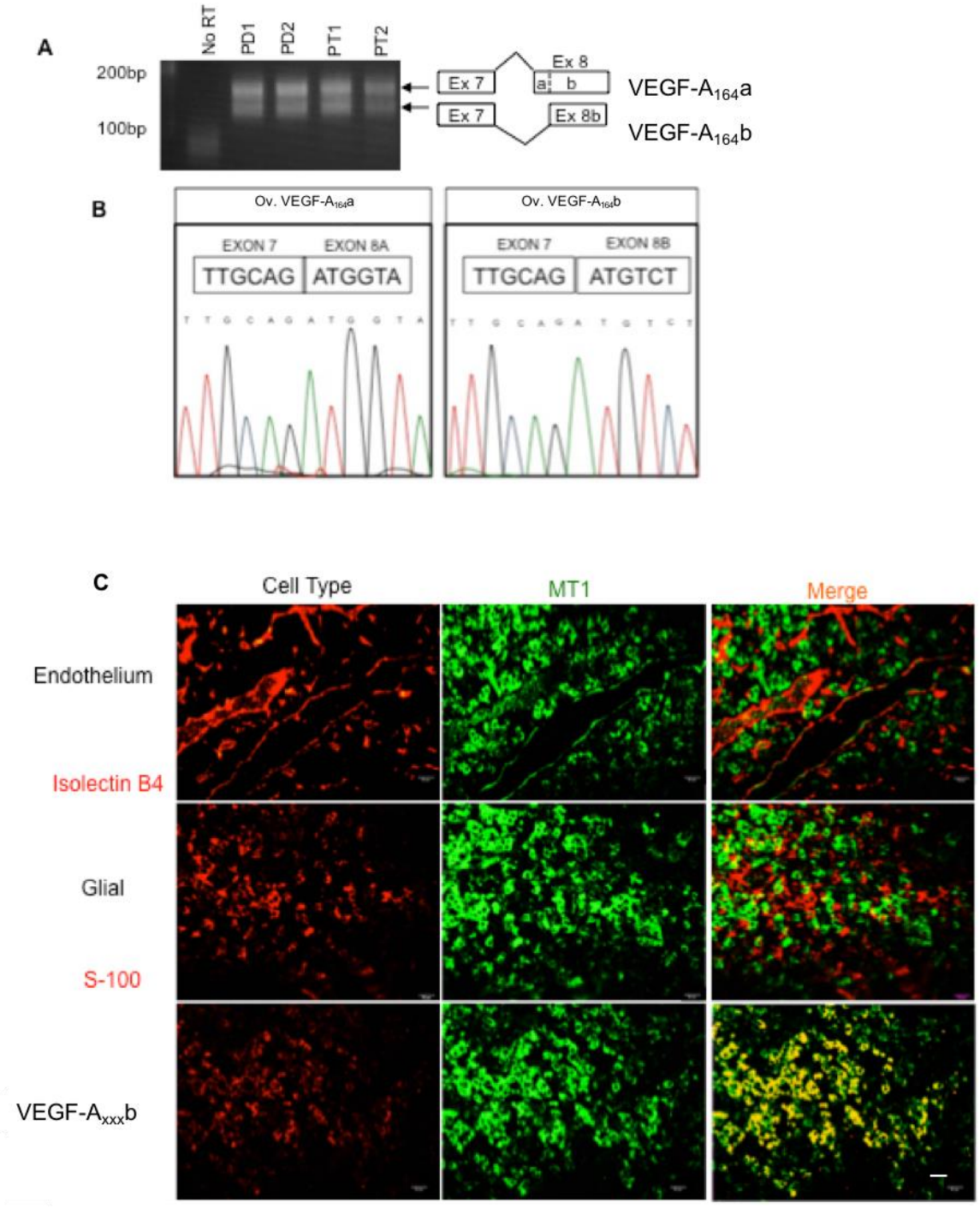
Total protein quantification

Measurement of total protein was determined using the BioRad Protein Assay. Each sample was measured in triplicate by adding 10µl per well (from 1:10 to 1:200 dilution, dependent on extract source) to a 96-well protein assay plate (BD Falcon). Serial dilutions of BSA (1000, 500, 250, 125, 62.5, 31.25, 15.125 and 0 ng/ml) were used to generate a standard curve. BioRad Protein Assay Dye Reagent (BioRad, Hemel Hemstead, UK) was diluted 1:5 in PBS, and 200µl per well added to samples. Finally, the concentration of total protein was measured using the Opsys MR plate reader (Dynex, USA) at a wavelength of 595nm and 490nm.

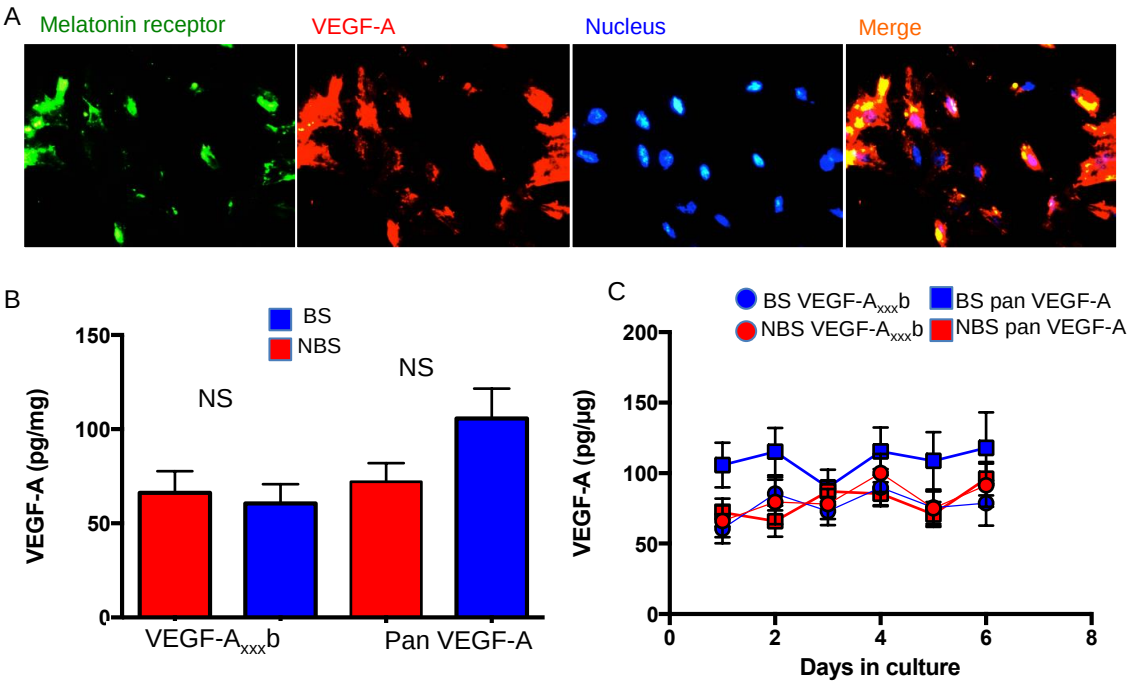
Protein extraction and VEGF-A/hormone assays

Protein extraction from cell cultures was carried out using RIPA buffer (50 mM Tris-HCl, pH 8.0, with 150 mM sodium chloride, 1.0% Igepal CA-630 (NP-40), 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulphate; Sigma) with an additional cocktail of proteinase inhibitors and stabilizers (1:50 protease inhibitor cocktail (Sigma). For both protein extracted from tissue and protein extracted from media, total VEGF-A and VEGF-$A_{xxx}b$ concentrations were determined by VEGF-$A_{xxx}b$ (DY3045, R&D Systems) and human VEGF-A (DY293B, R&D Systems) ELISAs. Protein was extracted from the media following the Trichloroacetic acid (TCA) precipitation procedure. Protein was extracted from tissue by homogenizing the tissue and adding a protease inhibitor cocktail, and RIPA buffer.

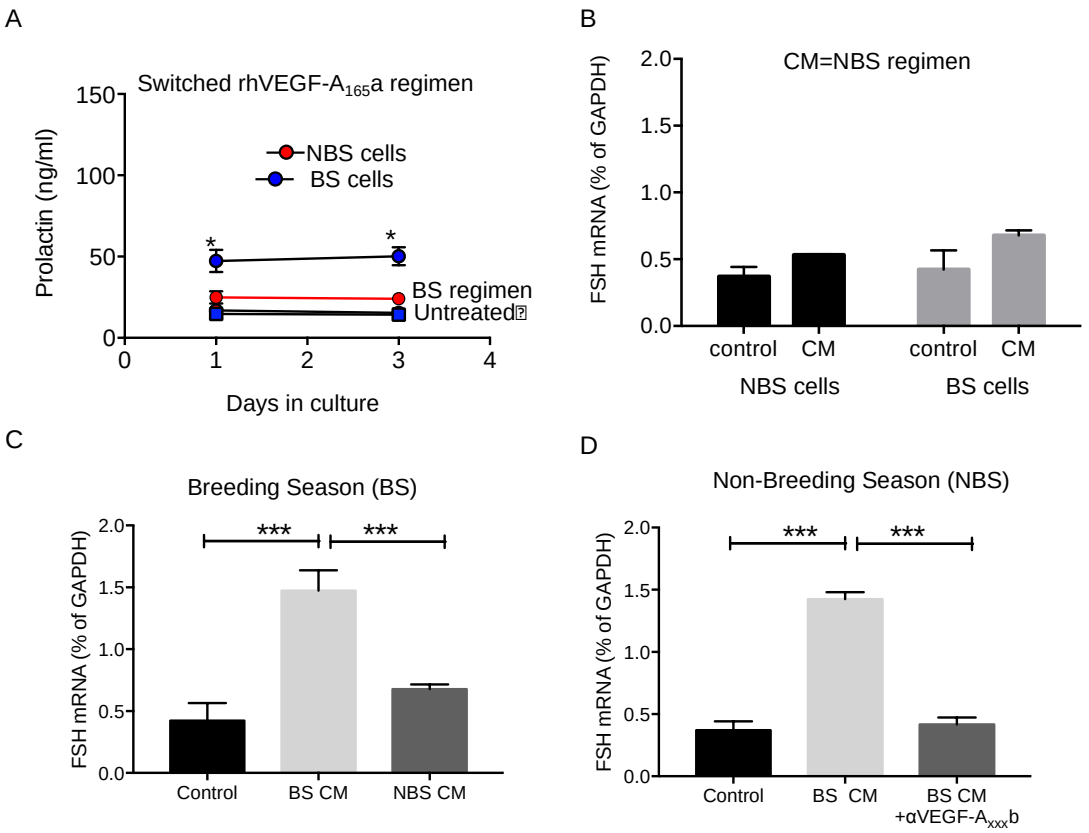
Both ELISA methods have been previously described [63-65]. A rh.VEGF-$A_{xxx}b$ positive control was included in triplicate for the human VEGF ELISA, allowing calculation of $VEGF\text{-}A_{total}$ concentration to compensate for reduced VEGF-$A_{xxx}b$ affinity, approximately 42%, as previously published [66]. The concentration of endogenous prolactin in culture wells following the application PD treatments was measured by RIA using purified ovine prolactin for standards and iodination provided by A. F. Parlow (University of California, Los Angeles, USA) and the NIDDK National Hormone and Peptide Program (USA), and an anti-ovine PRL antibody (ASMcN R 50) provided by A. S. McNeilly (Medical Research Council Human Reproductive Sciences Unit, Edinburgh, UK), as previously described [61]. A linear relationship was detected when the measured hormone concentration (ng／ml) was plotted against the concentration of diluted serum samples.



Supplementary Figure 1. Relates to figure 1. A. cDNA amplified from the pituitary pars distalis (PD) and pars tuberalis (PT) from ewes by primers that detect both exon 8a and exon 8b containing isoforms. Both bands were purified and sequenced. B Chromatogram of sequence of PCR products. Upper band (left) shows splicing from exon 7 to exon 8a. Lower band shows splicing from exon7 to exon 8b. C. MT1 receptor was co-localized with VEGF-Axxxb staining, but not glial cells or endothelium (Scale bar = 20□m).



Supplementary Figure 2. Relates to figure 2. A. Pars tuberalis (PT) cells were isolated from sheep in the breeding season (BS) and stained for the melatonin receptor, VEGF-A and Hoescht. B. VEGF levels from cells cultured in the absence of melatonin. There were no significant differences between VEGF-A levels from PT cells taken from the winter (blue, breeding season - BS), or summer (red, non-breeding season – NBS) ewes. C. VEGF levels did not alter over 6 days in primary culture (Scale bar = 20μm).



Supplementary Figure 3 Relates to figure 4. A. Pars distalis cells from the non-breeding season (NBS) treated with rhVEGF-A_{165}a in a breeding season (BS) regimen (red) do not produce prolactin. In contrast, cells from the BS treated with rhVEGF-A_{165}a in a NBS manner (blue) did produce prolactin. B. Conditioned media from pars tuberalis (PT) cells treated with a NBS melatonin regimen had no effect on FSH production in PD cells, irrespective of which season the cells were from. C. BS cells treated with BS, but not with NBS, PT conditioned media produced FSH mRNA. D. NBS cells treated with CM from PT cells exposed to a BS regimen of melatonin produced FSH. This was blocked by incubation with an antibody to VEGF-A_{xxx}b (□VEGF-A_{xxx}b). *=$p<0.05$ compared with untreated.

Supplementary Table 1. Primary antibodies

Peptide Target	Antigen Sequence	Species raised in	Manufacturer & Catalog code or reference	Working concentration
VEGF-A_{xxx}a	Raised against human pro-angiogenic isoforms (exon 8a)	Rabbit polyclonal	Produced in house (MVRL)	1mg/ml diluted 1:1000
VEGF=A_{xxx}b	Against human anti-angiogenic isoforms (exon 8b; 56/8)	Mouse monoclonal	Produced in house (MVRL)	2.5mg/ml diluted 1:100
Pan VEGF-A	Against all isoforms of human VEGF-A (A20)	Rabbit polyclonal	Santa Cruz Biotechnology; sc-152	200ug/ml diluted 1:100
VEGF receptor 2	Soluble extracellular human VEGF-R2	Mouse monoclonal	Abcam; ab9530	1mg/ml diluted 1:20
Folliculo-stellate cells (FSC)	Recombinant full length bovine S-100 protein	Rabbit polyclonal	Abcam; ab868	Concentration undetermined by manufacturer – dilution 1:1000
Melatonin receptor	C-terminus of human MT1-R	Goat polyclonal	Santa Cruz; sc-13186	200ug/ml diluted 1:100 to 2ug/ml
Prolactin	Raised against full length ovine prolactin	Rabbit polyclonal	Lifespan biosciences; LS-C124425	Unknown manufactures concentration; 1:5000 dilution
Proliferation marker	Proliferating cellular nuclear antigen (PCNA)	Mouse monoclonal	Invitrogen; 08-0110	2 ug/ml
Endothelial cell marker	Isolectin b4- against using human blood group B erythrocytes	Lectin from *Bandeiraea simplicifolia* - Isolectin B4 (BSI-B4), peroxidase conjugate, lyophilized powder	Sigma Aldrich; *L5391*	200ug/ml
Endothelial cell marker	Raised against murine CD31	Rabbit polyclonal	Abcam; ab28364	2ug/ml
Endothelial cell marker				

Supplementary Table 2. Secondary antibodies

Species raised	Species against	Colour (wavelength)	Company	Catalogue number
Goat	Mouse	Green (488)	Life technologies	A-10680
Donkey	Rabbit	Red (555)	Life technologies	A-31572
Streptavidin	NA	Green (488)	Life technologies	S11223
Streptavidin	NA	Red (555)	Life technologies	S21381

Supplementary Table 3. Primers

Name	Sequence
VEGF-A FWD 1	CAAATGTGAATGCAGACCAAAG
VEGF-A REV 1	TGTGTCAGTCTTTCCTGGTGA
VEGF-A FWD 2	CTCACCAAAGCCAGCACATAG
VEGF-A REV 2	GACACAGAACTACCCATAGCCG
VEGF-A FWD 3	CTCACCAAAGCCAGCACATAG
VEGF-A REV 3	ACACAGAACTACCCATAGCCG
VEGF-A exon 1 FWD	CGG TGGTACTTGAAAGAC
VEGF-A exon 8b REV	CAGAGTGGTCCTTTCTGACTGTGTCTTGCTGGGTATCGGCGGC
VEGF-A exon 7 FWD	ATAAAGCAAGGCAAGAAATCCCTG
VEGF-A exon 7 FWD2	GAAATCCCTGTGGGCCTTGCTAGA